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The cephalic sensory organs of *Acteon tornatilis* (Linnaeus, 1758) (Gastropoda Opisthobranchia) – cellular innervation patterns as a tool for homologisation*

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Abstract. Gastropoda are guided by several sensory organs in the head region, referred to as cephalic sensory organs (CSOs). This study investigates the CSO structure in the opisthobranch, *Acteon tornatilis* whereby the innervation patterns of these organs are described using macroscopic preparations and axonal tracing techniques.

A bipartite cephalic shield and a lateral groove along the ventral side of the cephalic shield was found in *A. tornatilis*. Four cerebral nerves can be described innervating different CSOs: N1: lip, N2: anterior cephalic shield and lateral groove, N3 and Nclc: posterior cephalic shield. Cellular innervation patterns of the cerebral nerves show characteristic and constant cell clusters in the CNS for each nerve.

We compare these innervation patterns of *A. tornatilis* with those described earlier for *Haminoea hydatis* (Staubach et al. *in press*). Previously established homologisation criteria are used in order to homologise cerebral nerves as well as the organs innervated by these nerves. Evolutionary implications of this homologisation are discussed.

Keywords. *Haminoea hydatis,* Cephalaspidea, axonal tracing, homology, innervation patterns, lip organ, Hancock´s organ.

1. INTRODUCTION

Gastropoda are guided by several organs in the head region which are assumed to have primarily chemo- and mechanosensory functions (Audesirk 1979; Davis & Matera 1982; Bicker et al.1982; Emery 1992; Chase 2000; Croll et al. 2003). In Opisthobranchia, these cephalic sensory organs (CSOs) present an assortment of forms including rhinophores, labial tentacles, oral veils, Hancock´s organs and cephalic shields. Recent investigations of CSOs in Opisthobranchia have focussed primarily on functional aspects (Croll 1983; Boudko et al. 1999; Croll et al. 2003) while homology of the different types of CSOs in different taxa has never been investigated in detail. We want to clarify their homology in separate evolutionary lineages so as to elucidate key questions regarding character evolution and phylogeny.

Acteon tornatilis belongs to the subgroup Acteonoidea, formerly ascribed to the basal Cephalaspidea (Odhner 1939, Burn & Thompson 1998). However, recent investigations have either excluded the Acteonoidea from the Opisthobranchia (Mikkelsen 1996) or proposed a sister group relationship of Acteonoidea and the highly derived Nudipleura (Vonnemann et al. 2005) thus, rendering the phylogenetic position of Acteonoidea within Opisthobranchia unsettled. Acteonoidea are characterised by the presence of a prominent cephalic shield. This structure is also present in Cephalaspidea and has been considered to be an apomorphie of the Cephalaspidea (Schmekel 1985). However, the structure of the cephalic shields differs considerably in Cephalaspidea and Acteonoidea with the latter possessing two distinct hemispheres while the cephalic shield in the Cephalaspidea possesses uniform structure. Therefore, common origin of both types of cephalic shields and thus homology is questionable. Further CSOs have been described in Acteonoidea and Cephalaspidea such as lip organ and Hancock´s organ (Rudman 1971a; Rudman 1971b; Rudman 1972a, b; Rudman 1972c; Edlinger 1980).

Since the presence of these organs in members of the genus *Acteon* has been disputed by different authors (Edlinger 1980; Schmekel 1985), absolute clarification is certainly necessary . The intention of this study is to describe the structure emphasizing the innervation of the CSOs in the acteonid *A. tornatilis*. Our descriptions focus on the cellular innervation patterns reconstructed for

the cerebral nerves using axonal tracing. In an earlier study (Staubach et al. *in press*) these cellular innervation patterns were shown to be more adequate in homologising cerebral nerves than ganglionic origins of nerves (Huber 1993). By comparising the innervation patterns in *A. tornatilis* to previously published data on *H. hydatis* (Staubach et al. *in press*), we want to survey whether the preliminary characteristic cell clusters in the central nervous system (CNS) of both taxa can be identified by homologising cerebral nerves across taxa. Based on the homologisation of the nerves innervating the CSOs we want to clarify if *A. tornatilis* has homologous structures to the CSOs of Cephalaspideans. It is our intent interest that we shed light on the phylogenetic position and evolutionary history of Acteonoidea within the Opisthobranchia for future studies.

2. MATERIALS AND METHODS

2.1. Specimens

A. tornatilis (Fig. 1A) were collected in the wild at St. Michel en Greve (Brittany, France). They were then stored alive at our lab in Frankfurt. Fourty specimens measuring a shell length between 15 and 20 mm were traced directly (5 to 15 days after collecting) and five were fixed for SEM.

2.2. Tracing studies

Animals were relaxed with an injection of 7 % magnesium chloride. The central nervous system consisting of the cerebral, pleural and pedal ganglia was removed and placed in a small Petri dish containing filtered artificial seawater (ASW; Tropic Marin, Rebic-Bielefeld; GERMANY). We then followed the proccdures from Croll & Baker (1990) for Ni^{2+}-lysine (Ni-Lys) tracing of axons. Briefly, the nerves of the right cerebral ganglion were dissected free from the connective tissue. The nerves were cut and the distal tip was gently drawn into a glass micropipette using suction provided by an attached 2.5 ml syringe. Subsequently, the saline in the micropipette was replaced by a Ni-Lys solution (1.9g $NiCl\text{-}6H_2O$, 3,5 g L-Lysine freebase in 20 ml double distilled H_2O). The preparation was then incubated for 12–24 hours at 8° C to allow transport of the tracer. The micropipette was then removed and the ganglia were washed in ASW three times. The Ni-Lys was precipitated by the addition of five to ten drops of a saturated rubeanic acid solution in absolute Dimethylsulfoxide (DMSO). After 45 minutes the ganglia were transferred to 4 % paraformaldehyde (PFA) and fixed for 4–12 hours at 4° C. Thereafter the ganglia were dehydrated in an increasing ethanol series (70/80/90/99/99% 10 minutes each), cleared in methylsalicylate and mounted on an objective slide dorsal side up in Entellan (VWR International) and covered with a cover slip. Ten replicates were prepared for each cerebral nerve of *A. tornatilis*. Samples with only a partial staining of the nerve were not used because of possible incomplete innervation patterns. Our criterion for a well-stained preparation was a dark blue stained nerve indicating intact axons (Fredman 1987). The Ni-Lys tracings were analysed by light microscopy (Leica TCS 4D). Camera lucida drawings were digitalised following the method of Coleman (2003) adapted for CorelDRAW 11. The somata in the innervation scemes occurs in all replicates. Somata only occurring in single samples are not considered part of the schematics. The axonal pathways are estimated over all replicates. Additionally, we tested for asymmetries making axonal tracings (n = 2 to 3) for each cerebral nerve of the left cerebral ganglion.

2.3. Scanning electron microscopy studies

The specimens were relaxed by an injection of 7 % $MgCl_2$ in the foot. Thereafter, the entire head region was dissected from the rest of the animal. The CSOs were fixed in 2,5 % glutaraldehyde, 1 % paraformaldehyde in 0,1M phosphate buffer (pH 7,2) at room temperature. For the SEM, the fixed CSOs were dehydrated through a graded acetone series followed by critical point drying (CPD 030, BAL-TEC). Finally, they were spattered with gold (Sputter-Coater, Agar Scientific) and examined with a Hitachi S4500 SEM. All photographs were taken using DISS (Digital Image Scanning System – Point Electronic) and subsequently adjusted for brightness and contrast with Corel PHOTO-PAINT 11.

3. RESULTS

3.1. Organisation and innervation of the cephalic sensory organs

A. tornatilis possesses a prominent bipartite cephalic shield (cs) in which each hemisphere of this shield is divided into an anterior and a posterior lobe (Figs. 1A and B). Eyes are embedded deeply within the tissue of the shield. Along the lateral margin of the anterior lobe of the cephalic shield a groove is present (Fig. 1B, 2A). Hidden under the cs and above the foot, the mouth opening is situated at the median frontal edge (Fig. 2B) surrounded by the lip (not visible in Figure 2B). We found four nerves innervating the CSOs (Fig.1B). The N1 (Nervus oralis) provides innervation to the lip and small median parts of the anterior cephalic shield. The bifurcated N2 (Nervus labialis/labiotentacularis) innervates the complete anterior cephalic shield whereby the groove at the ventral anterior lobe of the cephalic shield is especially innervated. The small N3 (Nervus tentacularis/rhinophoralis) innervates a little re-

gion of the posterior cephalic shield. The Nclc (Nervus clypei capitis) innervates the largest hind part of the posterior cephalic shield. We could not detect a lip organ (Fig. 2B), which according to EDLINGER (1980) should comprise two small lobes on the cephalic shield above the mouth. A Hancock's organ described by EDLINGER (1980) for *A. Tornatilis*, here a folded structure separated from the cephalic shield was likewise not found in the present study.

3.2. Tracing studies

By conducting the axonal tracing studies we were able to reconstruct cellular innervation patterns for the four cerebral nerves of *A. tornatilis*. Ten replicate tracings were performed each for the N1 N2, N3 and Nclc using only the nerves of the right cerebral ganglion. The characteristic patterns of labelled somata for all nerves are shown in Figure 3A-D, including the approximate pathways of the stained axons. The identified clusters were named with abbreviations signifying the ganglion in which they are located, the nerve filled and a number indicating the order of their description (for example, Cnlc3: Cerebral Nervus labialis cluster 3). Nerve cells are grouped in clusters on the basis of their close positioning in the ganglia and the tight fasciculation of their axons projecting into the filled nerve. Asymmetries for tracings of the left nerves could not be detected.

For the N1 (n = 10) we identified six cerebral clusters (Cnoc1-6) and one pedal cluster (Pdnoc1) in each sample (Fig. 3A). The variation between the samples was restricted to very few somata in some clusters. The cerebral clusters were distributed over the whole cerebral ganglion. The pedal cluster Pdnoc1 was located on the anterior margin of the pedal ganglion above the pedal commissure. The innervation pattern of the N2 (n=10) consists of five cerebral clusters (Cnlc1-5) and three pedal clusters (Pdnlc1-3) (Fig. 3B). The cerebral clusters show distinct spatial separations and are easy to identify. The third traced cerebral nerve (n = 10) was the N3. Six cerebral (Cnrc1-6) and three pedal clusters (Pdnrc1-3) were identified (Fig. 3C). We found an additional single cluster (*Cclnrc1*) and a single soma in the left cerebral ganglion (see arrows in Fig. 3C). The contralateral cluster was located at the base of the N2 whereas the single soma was found at the root of the cerebral commissure. We observed slight intraspecific variability between the ten samples which amounted only to very few somata in some clusters. In the Nclc, the innervation (n = 10) pattern consisted of five cerebral clusters (Cncc1-5) and a single soma at the lateral margin of the cerebral ganglion above the pedal connective (Fig. 3D). Additionally we found four pedal clusters (Pdncc1-4). The Nclc had the highest amount of pedal clusters in all investigated nerves. The number of pedal somata, however, was comparable to the number of pedal somata for the N2 innervation pattern (Fig 3B).

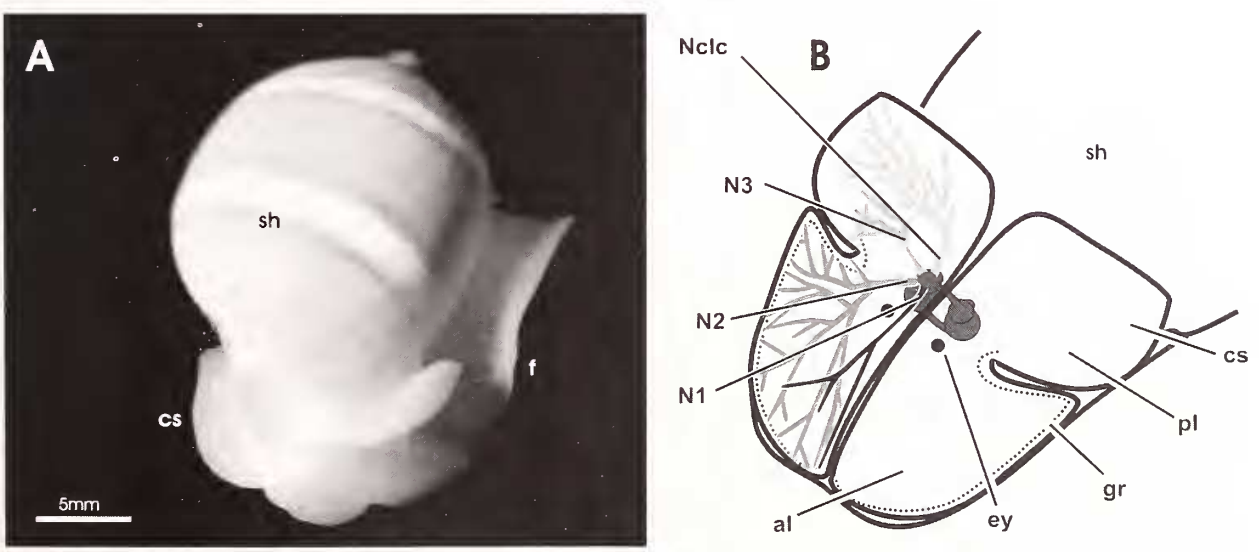

Fig. 1. **A:** Photograph of *Acteon tornatilis* with the cephalic shield visible. **B:** Schematic illustration of the CNS, the four cerebral nerves (excluding the optical nerve) and the cephalic sensory organs of *Haminoea hydatis* and *Acteon tornatilis*. Only the right cerebral nerves are shown. N1 Nervus oralis, N2 Nervus labialis, N3 Nervus rhinophoralis, Nclc Nervus clypei capitis, ey eye, gr groove, al anterior lobe, pl posterior lobe, sh shell, cs cephalic shield, f foot.

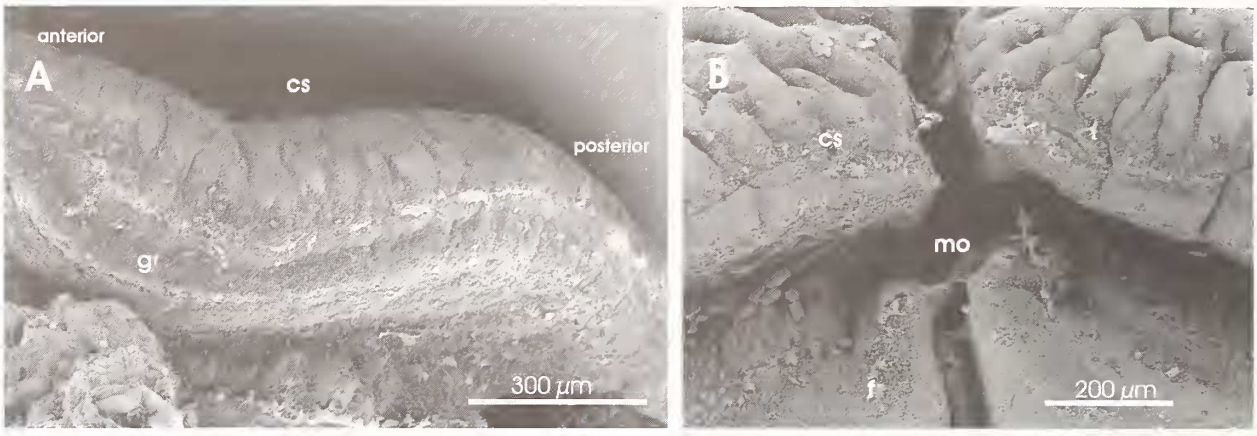

Fig. 2. A. Lateral SEM photography of the groove at the ventral surface of the cephalic shield of Acteon tornatilis. cs cephalic shield, gr groove. B. Frontal SEM photography of the mouth region of *Acteon tornatilis*. cs – cephalic shield, mo – mouth, f – foot.

4. DISCUSSION

The present study demonstrates the constancy of nervous structures in the opisthobranch mollusc. Throughout our investigation of several individuals of the aeteonid, *Acteon tornatilis* we found uniform innervation patterns of the head region via four cerebral nerves, which can be attributed to characteristic neuronal cell clusters in the CNS. These cellular innervation patterns in *A. tornatilis* show an extremely high congruence with the cellular innervation patterns described for the four cerebral nerves of *Haminoea hydatis* (Staubach et al. in press).

In the N1, the number of cerebral clusters as well as the position of these clusters to each other is the same in *A. tornatilis* and *H. hydatis*. However, we found some differences in the size and number of somata when comparing both species. Additionally, we could not detect a pleural, a parietal and a pedal cluster in *A. tornatilis,* which were described for *H. hydatis*. This may be due to the differences in the peripheral innervation area of the N1. In *A. tornatilis* it only provides for the lip and very small parts of the median cephalic shield whereas in *H. hydatis,* it innervates the lip and large parts of the anterior cephalic shield. For the second nerve, the N2 (Nervus labialis), we nearly found no differences between the presence and distributions of the cell clusters for both species. The only ostentatious difference was the lack of a single pedal soma and its contra-lateral analogue in *A. tornatilis*. In the Nele (Nervus clypei capitis), the difference between the two species was also reduced to the presence of a single cerebral soma in *A. tornatilis*. In contrast to the three nerves described above, we found a prominent difference in the structure of the N3 when comparing *Acteon* and *Haminoea*. On the other hand, in *H. hydatis* the N3 terminates in a rhinophoral ganglion. Such a ganglion is missing in *A. tornatilis*. Hence, we expected considerable differences in the cellular innervation patterns for the N3 of these species. However, these differences were marginal and only amounted to the lack of one single cell soma in the cerebral ganglion of *A. tornatilis*. This implies that basic innervation patterns of the N3 are probably the same in both species. Additional functions of the N3 processed in the rhinophoral ganglion can be proposed for *H. hydatis*. These functions are probably related to the Hancock´s organ, which is innervated by nerves originating in the rhinophoral ganglion (Staubach et al. *in press*). We were unable to locate such an organ in *A. tornatilis* in contrast to earlier descriptions (Edlinger 1980).

Upon comparing the innervation patterns presented here for *A. tornatilis* with those for *H. hydatis* (Staubach et al. *in press*) we find constant features of these patterns across species. This is congruent with other findings that neuronal structures in the central nervous system of molluscs and other invertebrates seem to be highly conserved (Croll 1987; Arbas 1991; Hayman-Paul 1991; Kutsch & Breidbach 1994; Newcomb et al. 2006). Hence, we postulate the N1 of *A. tornatilis* to be homologous to the N1 (Nervus oralis) described by Huber (1993) for Cephalaspideans. Additionally, we postulate homologies of the N2 and the N3 of *A. tornatilis* to the N2 (Nervus labialis) and N3 (Nervus rhinophoralis) of Chepalaspideans. This is congruent to the assumption of Hoffmann (1939) that the c3 (after Vayssière 1880) of *H. hydatis* represents the Nervus labialis and the c4 represents the Nervus tentacularis, here a synonym for the Nervus rhinophoralis (Huber 1993). Our data cannot support Edlinger's (1980) description of independent nerves for the lip organ (N1 after Edlinger 1980) and the anterior Hancock's organ (N2 after Edlinger 1980). The Nele of *A. tornatilis* also seems to be homologous to the Nele of Cephalaspideans (Huber

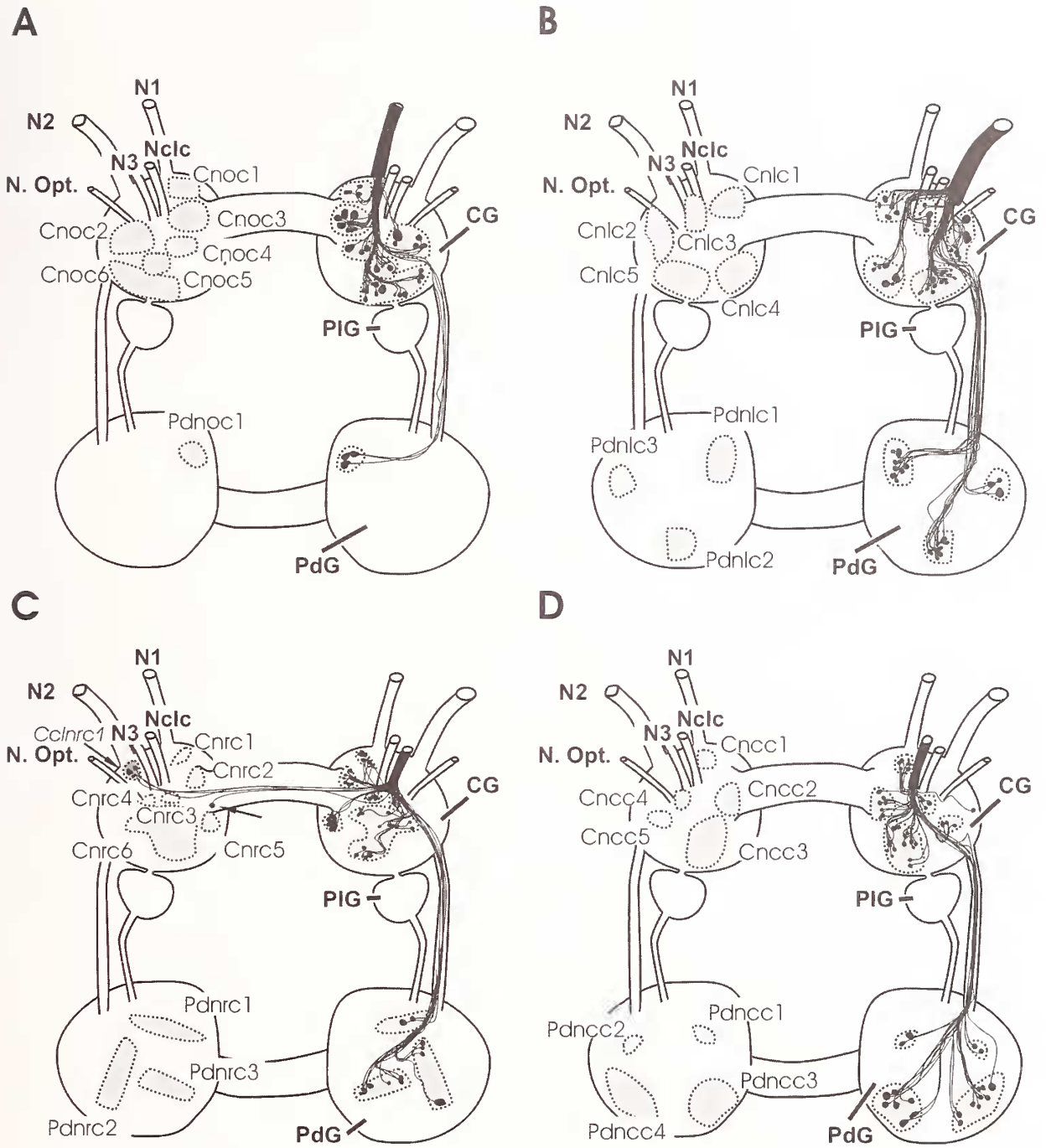

Fig. 3. Schematic outline of ccll clusters providing the N1 **(A)**, N2 **(B)**, N3 **(C)** and Nclc **(D)** of *Acteon tornatilis*. The size and position of the somata were digitalized from a camera lucida drawing, the distribution of the axons are averaged from all replicates. N1 Nervus oralis, N2 Nervus labialis, N3 Nervus rhinophoralis, Nclc Nervus clypei capitis, N. opt. Nervus opticus, CG cerebral ganglia, RhG rhinophoral ganglia, PlG pleural ganglia, PdG pedal ganglia.

1993). Hoffmann (1939) described the same nerve as the Nervus proboscidis. We define this nerve however, as Nervus clypei capitis according to Huber (1993).

Considering the homologisation of the cerebral nerves in light of their neurological origin, neuro-anatomics and nervous innervation patterns, we postulate hypotheses of homologies respective of the organs innervated by these nerves. Thus, we consider the lip of *A. tornatilis* to be homologous to the lip of Cephalaspideans (Huber 1993) since both organs are innervated by the N1. The same holds true for the small median parts of the cephalic shield in *Acteon* and the anterior cephalic shield of *Haminoea.* We could not find a lip organ in *A. tornatilis* as described by Edlinger (1980), but we detected a groove at the ventral side of the anterior cephalic shield. This groove is innervated by the N2 as is the lip organ of Cephalaspideans (Huber 1993). Therefore, we postulate this groove to be homologous to the lip organ. This hypothesis is also supported by data on immunoreactivity against several neurotransmitters. In the groove of *A. tornatilis* as well as in the lip organ of *H. hydatis,* characteristic sub-epidermal sensory neurons containing catecholamines could be found in high density indicating that both organs are involved in contact chemoreception (S. Faller, Frankfurt, pers. comm. 2007).

The N2 of *Haminoea* is divided into two branches which are described as two single nerves by Edlinger (1980). The first or inner branch provides the lip organ as described earlier. The second, outer branch is related to the anterior Hancock´s organ (Edlinger 1980; Huber 1993). In *Acteon* we also found two branches of the N2: the inner one providing the largest part of the groove whereas the outer branch is restricted to a small region between the anterior and posterior lobe of the cephalic shield. Therefore, this latter region may be homologous to the anterior Hancock´s organ of *H. hydatis* and not to the posterior Hancock´s organ as described by Edlinger (1980).

The N3 of *A. tornatilis* provides a large part of the posterior cephalic shield but no identifiable posterior Hancock´s organ. Additional immunohistochemical and ultrastructural investigations could also not detect a posterior Hancock´s organ in *A. tornatilis* (S. Faller, Frankfurt, pers. comm. 2007; Göbbeler & Klussmann-Kolb *in press*). The posterior parts of the cephalic shields in *Acteon* and *Haminoea* are probably equally homologous as both where innervated by the Nelc.

The lack of a posterior Hancock´s organ in *A. tornatilis* might be due to three different reasons: 1. the ancestor of *A. tornatilis* never had a posterior Hancock´s organ; 2. the posterior cephalic shield of *A. tornatilis* may be a homologous structure to the posterior Hancock´s organ of *H. hydatis;* and 3. the posterior Hancock´s organ has secondarily been reduced in *A. tornatilis.*

The first hypothesis is rather implausible since we found a distinct N3 with conserved cellular innervation patterns in the central nervous system. If the ancestor of A. tornatilis never had a posterior Hancock´s organ, this nerve and associated neural structures should be lacking. Moreover, a Hancock´s organ has been described for other Acteonoidea (Rudman 1971a, b; Rudman 1972a, b; Rudman 1972c). If we consider the second explanation for lack of a posterior Hancock´s organ in *A. tornatilis,* we imply that the posterior cephalic shield in this species, innervated by the N3, presents a sensory organ as the Hancock´s organ in Cephalaspidea. However, immunohistochemical and ultrastructural investigations of the respective epithelia in *A. tornatilis* do not indicate a sensory function at all (S. Faller, Frankfurt, pers. comm. 2007; Göbbeler & Klussmann-Kolb *in press*). We reject this hypothesis of homology of the posterior cephalic shield in *A. tornatilis* and posterior Hancock´s organ in *H. hydatis* since we found no evidence for a function of the posterior cephalic shield as an olfactory sensory organ. Moreover, the posterior cephalic shield is mostly innervated by the Nelc and not by the N3. The third hypothesis regarding the reduction of a Hancock´s organ seems to be the most plausible when the habitat and the food sources of *A. tornatilis* in comparison to *H. hydatis* are considered. The posterior Hancock´s organ is believed to be an olfactory sensory organ (Audesirk 1979; Emery 1992). *H. hydatis* feeds on green algae which occur in patches in open water whereas *A. tornatilis* is a predator of soft invertebrates living up to ten centimeters in solid sand (Fretter 1939; Yonow 1989; own investigations). In such an environment, an olfactory sensory organ is not plausible since olfaction or distance chemoreception is generally associated with water currents, which are not substantial in a sandy substrate habitat. Here, a contact chemoreceptor, which is located near the edge of the cephalic shield is more plausible. This we witnessed in *Acteon tornatilis* via its display of a potentially chemoreceptive groove along the lateral margin of the anterior cephalic shield.

This assumption of secondary reduction of the Hancock´s organ in the endobenthic *A. tornatilis* is also supported by the fact that a Hancock´s organ has been described for other epibenthic Acteonoidea (e. g. *Bullina, Micromelo, Hydatina*) (Rudman 1971a, b; Rudman 1972a, b; Rudman 1972a,b,c).

Despite all discussion, homology of the described Hancock´s organs to those in Cephalaspidea cannot undoubtedly be proposed at this stage, particularly since data on innervation patterns in these acteonids are lacking to date. Moreover, current phylogenetic hypotheses (Grande et al.

2004; Vonnemann et al. 2005) regarding Opisthobranchia propose an independent origin of Acteonoidea and Cephalaspidea, indicating convergent development of these sensory organs in both evolutionary lineages. Further studies will have us utilizing cellular innervation patterns for CSOs in order to compare several taxa while homologising the different types of CSOs in Opisthobranchia. This procedure will enable us to glean a better understanding of the evolution of these organs.

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